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Insulin Resistance is Associated with Higher Intramyocellular Triglycerides in Type I but not Type II Myocytes Concomitant with Higher Ceramide Content

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ABSTRACT

OBJECTIVE-We tested the primary hypotheses that sphingolipid and diacylglycerol (DAG) content is higher within insulin resistant muscle and that the association between intramyocellular triglycerides (IMTG) and insulin resistance is muscle fiber type-specific. **RESEARCH DESIGN AND METHODS-**A nested case-control analysis was conducted in 22 obese (BMI >30 kg/m^2) women who were classified as insulin resistant (IR; n = 12) or insulin sensitive (IS; n = 10), determined by hyperinsulinemic euglycemic clamp (>30% greater in IS compared to IR, P <0.01). Sphinoglipid and DAG content was determined by HPLC/MS/MS. Fiber type-specific IMTG content was histologically determined. Gene expression was determined by qPCR. **RESULTS-**Total (555±53 vs. 293±54 pMol/mg protein, P =0.004), saturated (361±29 vs. 179±34 pMol/mg protein, P =0.001) and unsaturated (198±29 vs. 113±66 pMol/mg protein, P =0.034) ceramides were higher in IR compared to IS. DAG concentrations, however, were similar. IMTG content within type I myocytes, but not type II myocytes, was higher in IR compared to IS (P =0.005). Insulin sensitivity was negatively correlated with IMTG within type I myocytes (R = -0.51, P =0.026), but not with IMTG within type II myocytes. The proportion of type I myocytes was lower (41% vs. 59%, P <0.01) in IR. Several genes involved in lipid droplet and fatty acid metabolism were differentially expressed in IR compared to IS. **CONCLUSIONS-**Human skeletal muscle insulin resistance is related to greater IMTG content in type I, but not type II myocytes, to greater ceramide content, and to alterations in gene expression associated with lipid metabolism.

Skeletal muscle insulin resistance is a key factor in the development of type 2 diabetes mellitus (T2DM), although the exact mechanisms that underlie insulin resistance in humans remain elusive. Considerable efforts have been made in the past several decades to better understand the role of impaired fatty acid metabolism in skeletal muscle insulin resistance (1, 2). Many earlier studies, both in animal models (3) and in humans (4, 5) indicated that intramyocellular triglyceride (IMTG) accumulation was associated with insulin resistance, thereby providing a potential link between dysregulated fatty acid metabolism and insulin resistance. However, it has become increasingly apparent that IMTG do not directly confer insulin resistance but rather, under certain circumstances, likely provide a surrogate for other bioactive lipid metabolites within muscle such as sphingolipids (including ceramide) and diacylglycerol (DAG) (4, 6).

Studies in animal and cell culture models indicate that sphingolipids and DAG can be key mediators of insulin resistance (7-9). However, a recent study of acute hyperlipidemia in rodents indicates that ceramide and DAG content are not associated with muscle insulin resistance (10). Furthermore, studies conducted in humans do not provide a firm consensus as to whether ceramides or DAGs are associated with muscle insulin resistance (11). Some studies indicate that muscle ceramide content is elevated in obese compared to lean individuals (12, 13), or that increases in muscle ceramide content as a result of lipid infusion (15), or conjugated linoleic acid supplementation (16), were associated with decreased insulin sensitivity. Endurance training in obese subjects reduced muscle ceramide content with a concomitant improvement in insulin sensitivity (17, 18). In contrast, a recent study by Skovbro et al. found that total muscle ceramide content was similar in subjects despite a wide range of insulin sensitivity (19). Also refuting a role for ceramide in human muscle insulin resistance, Itani et al., reported that lipid-induced

insulin resistance did not alter ceramide content (14).

The role of muscle DAG content in insulin resistance is also unclear. Muscle DAG content has been shown to be elevated in obese and T2DM subjects (20) and increased following a 6-hr lipid infusion which reduced insulin sensitivity (14). In contrast, IMTG, but not DAG content in muscle, was reported to be elevated in obese diabetic compared to obese nondiabetic subjects (21). Therefore, in order to help address the controversy in the literature surrounding the role for specific lipid metabolites in insulin resistance, we conducted comprehensive mass spectrometry-based DAG and sphingolipid profiling in human muscle insulin resistance while accounting for factors well known to influence insulin sensitivity, namely obesity and physical activity.

Quantification of intramyocellular lipids in humans is further complicated by a heterogenous myocyte or fiber composition. Type I myocytes have a greater capacity for oxidative phosphorylation (22, 23), and typically can contain two-to-three times more IMTG than type II myocytes (22, 24, 25). Furthermore, type I myocytes have been reported to be more insulin sensitive than type II (26, 27). However, it is not clear, insulin sensitivity is related to differences in myocyte specific IMTG content, nor is it known whether sphingolipid or DAG content is related to muscle cell type. We tested the primary hypotheses that sphingolipid and DAG content is higher within insulin resistant muscle and that type I myocyte IMTG content is specifically related to insulin resistance. We also explored whether differences in the expression of several genes related to lipid metabolism could play a role in insulin resistance associated with IMTG accumulation.

RESEARCH DESIGN AND METHODS

Study population. Subjects for this nested case-controlled study were selected from ongoing clinical investigations of skeletal muscle insulin resistance. Only female subjects were included to provide a gender-controlled analysis. Women aged 60–75 yrs were recruited though advertisements in the Pittsburgh, PA area. A brief phone screening initially determined eligibility for study participation. Potential subjects who were determined to be sedentary (exercise ≤1 day/wk), weight stable (±3kg in the previous 6mo), with a body mass index (BMI) >30 kg/m^2, and nonsmokers were further evaluated at the Clinical Translational Research Center (CTRC) at the University of Pittsburgh. Exclusionary criteria included uncontrolled hypertension (>150/95 mmHg), anemia (Hct<34%), elevated liver enzymes (25% above normal), proteinuria, or hypothyroidism (sensitive TSH 8mIU/l). Potential subjects were also excluded if they reported taking chronic medications known to affect glucose homeostasis. Following the medical screening, potential subjects then completed a 2-h, 75-g oral glucose tolerance test (OGTT) to determine glucose tolerance status. Subjects with T2DM determined by fasting glucose >126mg/dl were excluded. All subjects gave written consent to the protocol, which was approved by the University of Pittsburgh's Institutional Review Board.

Insulin sensitivity. Insulin sensitivity was determined by a 4-hr hyperinsulinemic (40 mU/m^2/min) euglycemic clamp and [6,6-$^2H_2$] glucose infusion as previously described (28). Briefly, subjects reported to the CTRC the evening prior to the clamp and following a 48-hr period in which they refrained from any vigorous physical activity. A continuous intra venous infusion of insulin (Humulin; Eli Lilly, IN) was begun at ~7AM, and euglycemia (target 90mg/dl) was maintained using an adjustable infusion of 20% dextrose. To measure rate of insulin stimulated glucose disposal and residual endogenous glucose production (EGP), a primed

(0.22 μmol/kg), continuous (17.6 μmol/kg/min) infusion of [6,6-$^2H_2$] glucose was administered (29). [6,6-$^2H_2$] glucose enrichment in plasma was determined by gas chromatography/mass spectrometry (6890 Network/5973 Series; Agilent, Santa Clara, CA) as previously described (30). Plasma glucose was measured by the glucose-oxidase method (2300 Stat Plus; Yellow Springs Instruments, Yellow Springs, OH). Fasting plasma insulin concentration was determined by an enzyme linked immunosorbent assay (Millipore, Billerica, MA). Rate of glucose disposal (R_d) was calculated using the tracer: tracee method with a modified version of the Steele et al., equation (31)

Muscle biopsy. Percutaneous muscle biopsy samples were obtained in the fasted state prior to the glucose clamp, as described previously (32). Briefly, muscle biopsies were obtained from the vastus lateralis, 15cm above the patella. A portion of the specimen (~30mg) was snap frozen in liquid nitrogen and stored at -80°C for sphingolipid and diacylglycerol analysis. A second portion (~20mg) was snap frozen and stored at -80°C for gene expression analysis. A third portion of the specimen for histochemistry was mounted on a small piece of cork with mounting medium (Shandon Cryochrome; Thermo Electron, Pittsburgh, PA), frozen in isopentane cooled with liquid nitrogen for 2–3 min (-160°C), and then placed into liquid nitrogen. All samples were stored at -80°C until analysis.

Analysis of sphingolipid and diacylglycerol species. Briefly, liquid nitrogen-frozen samples (~30mg) were homogenized in ice-cold buffer (250mM sucrose, 25mM KCl, 50mM Tris, and 0.5mM EDTA, pH 7.4). Total content as well as the various molecular species of diacylglycerol and sphingolipid were measured by high-performance liquid chromatography-tandem mass

spectrometry as previously described in detail (33).

Analysis of non-esterified plasma free fatty acids. Plasma free fatty acid concentrations were determined by gas chromatography with flame ionization detection (6890N; Agilent, Santa Clara, CA) using heptanoic acid as an internal standard. Free fatty acids were derivatized with dimethylamine using Deoxo-Fluor reagent (Sigma-Aldrich, St. Louis, MO).

Histochemical analysis for fiber type and IMTG content. Histochemical analysis was performed on serial sections using a modified version of methods previously used in our laboratory (32). Briefly, mounted biopsy samples were sectioned (10μm) on a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, PA) at -20°C and placed on a glass slide. Sections were then stained in a filtered solution of Oil Red O (300mg/ml in 36% triethylphosphate) for 30 minutes at room temperature. Thereafter, sections were incubated with primary antibodies for anti-human myosin heavy chain (MYH)7 (type I myocytes) and MYH2 (type IIa myocytes) overnight at room temperature and subsequently incubated with fluorescein (FITC) (type IIa myocytes) and Rhodamine (type I myocytes) conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Type IIx fibers remained unstained. Images were visualized using a Leica microscope (Leica DM 4000B; Leica Microsystems, Bannockburn, IL), digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and analyzed using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, NY). Oil red O staining intensity and cross sectional area were determined in Type I and Type II myocytes. Analysis is based on ~200 fibers per section (range 130-350).

Gene expression analysis. Gene expression analysis was carried out in a subset of subjects from each group, based on availability of vastus lateralis biopsy specimen (6 IR and 6 IS). Total RNA was isolated using Trizol reagent (Invitrogen; Carlsbad, CA). Isolated RNA was then further purified using a silicon-membrane spin column including an on-column DNase I treatment (Qiagen, Valencia, CA). Total RNA was reverse transcribed using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (SABiosciences, Frederick, MD). Gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR green based custom RT^2 Profiler PCR Arrays (SABiosciences, Frederick, MD). qRT-PCR was performed on an ABI prism 7900 (Applied Biosystems, Foster City, CA). Baseline Ct and threshold were determined and set for all samples. The relative level of mRNA expression for each gene in each sample was normalized to the average expression of β-2-microglobulin, hypoxanthine phosphoribosyltransferase (HPRT1), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and beta actin (ACTB), and expressed as $2^{-\Delta Ct}$.

Body composition and peak aerobic capacity. Total body fat and lean mass were assessed using dual-energy X-ray absorptiometry (GE Lunar Prodigy and Encore 2005 software v9.3). Subjects performed a VO_2 peak test on an electronically braked cycle ergometer to determine aerobic fitness (Sensor Medics, Yorba Linda, CA) as previously described (34).

Statistical analysis. Group comparisons were made by an independent two-tailed Students t-test. Correlations between variables were determined by Person's correlation co-efficient. All data are mean ± SEM unless otherwise stated. Statistical significance was set at $P<0.05$.

RESULTS

Subject characteristics. Ten insulin sensitive (IS) and 12 insulin resistant (IR) women were matched for age, body mass, BMI and physical fitness (VO_2 peak) (Table 1). There was no significant group difference in fasting plasma glucose, insulin or free fatty acid levels. However, by design, R_d during the hyperinsulinemic euglycemic clamp was greater ($P<0.01$) in the IS when compared to the IR group (Table 1). 2-hr plasma glucose levels during the OGTT were also significantly higher in the IR group compared to the IS group ($P=0.016$). The IR (n=6) and IS (n=6) subgroups for gene expression analysis had similar characteristics as their respective groups overall (data not shown).

Muscle fiber distribution. Type I and II myocyte content and myocyte average cross sectional area are presented in Table 2. The IS group had a higher proportion of type I myocytes ($P=0.002$) and a lower proportion of type IIa ($P=0.014$ and type IIx ($P=0.02$), than the IR group. When data from both groups were combined, there was a higher proportion of type IIa compared to type IIx myocytes ($P=0.008$). No differences were observed in myocyte average cross sectional area. With both groups combined, there was a significant positive correlation between the proportion of type I myocytes and insulin sensitivity and a negative correlation between the proportion of type II myocytes (type IIa and IIx combined) and insulin sensitivity (Table 3).

Muscle content of sphingolipid and diacylglycerol. The data for muscle content of sphingolipid and diacylglycerol species is presented in Figure 1. We observed higher total levels of ceramide (555±53 vs. 293±34 pMol/mg protein, $P=0.004$), total unsaturated ceramide (198±29 vs. 114±21 pMol/mg protein, $P=0.034$), and total saturated ceramide (361±29 vs.

179±34 pMol/mg protein, P =0.001) in the IR group, compared to the IS group. We also observed higher levels of specific ceramide species containing the following fatty acids; myristic (C14:0) (4.0±0.8 vs. 1.9±0.4 pMol/mg protein, P =0.044), palmitic (C16:0) (58±14 vs. 18±5 pMol/mg protein, P =0.023), and stearic (C18:0) (72±10 vs. 40±7 pMol/mg protein, P =0.02) in IR compared to IS muscle. There also tended to be greater content of sphingolipids containing palmitoleic (C18:1) (43±11 vs. 19±3 pMol/mg protein, P =0.077) and lignoceric (C24:0) (201±25 vs. 123±29 pMol/mg protein, P =0.06) acids, and sphingosine (3.4±0.6 vs. 2.2±0.3 pMol/mg protein, P =0.08) in IR muscle. When data were combined from both groups we observed a significant negative correlation between total unsaturated, total saturated, and total ceramide content and insulin sensitivity (Table 3). Total ceramide (R =0.517, P<0.05) and total saturated ceramide (R =0.523, P<0.05) were positively correlated with Type II myocyte percentage. However, no significant differences in total or individual diacylglycerol species were observed between IS and IR subjects.

Myocyte-specific IMTG content. Type I and II myocyte-specific IMTG content are shown in Figure 2. Total muscle IMTG was greater in the IR group compared to the insulin sensitive group (P =0.009). Type I myocyte-specific IMTG content was also greater in the IR group compared to the IS group (P =0.005). There was no difference in type II myocyte-specific IMTG content between IS and IR groups, nor was IMTG content different in IR within subtypes of type IIa and type IIx myocytes. When both groups were combined we observed a significant negative correlation between type I myocyte IMTG content and insulin sensitivity whereas there was no relationship between insulin sensitivity and type II myocyte (type IIa and IIx combined) IMTG content (Figure 2).

Muscle gene expression. The gene expression data for IR and IS sub-groups is presented in Figure 3. Expression levels of key genes, including facilitated glucose transporter member 4 (GLUT4), insulin receptor substrate (IRS)1 and IRS2 were lower in IR, indicating insulin resistance in this group at the tissue level in accord with their insulin resistance assessed in vivo. The expression of genes relating to oxidative phosphorylation transcription factors, fatty acid metabolism, ceramide metabolism, and lipid droplet associated proteins were examined. We found lower expression of the transcription factors peroxisome proliferator-activated receptor (PPAR) α and PPARγ in the IR group compared to the IS group. Also, expression of PPARγ coactivator 1α (PGC-1α) and nuclear respiratory factor 1 (NRF1) tended to be lower in IR muscle. Furthermore, we observed a lower expression of carnitine palmitoyltransferase 1B (CPT1B) and malonyl-CoA decarboxylase (MLYCD), genes that are related to fatty acid metabolism. Gene expression of lipid droplet associated proteins hormone-sensitive lipase (HSL), adipose differentiation-related protein (ADRP), adipose triglyceride lipase (ATGL), and synaptosomal-associated protein 23 (SNAP23) was lower in the IR group compared to IS. Interestingly, the expression of genes relating to ceramide metabolism was not different between groups. The expression of tumor necrosis factor alpha (TNFα) tended to be higher in the IR group compared to the IS group ($P = 0.09$).

DISCUSSION

Despite a preponderance of evidence from cell culture and animal models suggesting a role for both ceramide and diacylglycerol in skeletal muscle insulin resistance (7, 8), there is actually no consensus as to whether this is relevant to human insulin resistance (12-14, 16, 18). This study was conducted for three purposes: (a) to determine whether diacylglycerol or

sphingolipid species are elevated in IR human skeletal muscle; (b) to investigate whether the associations between intramyocellular lipids are dependent on muscle fiber type; and (c) to explore whether the skeletal muscle expression of several key genes relating to lipid metabolism are altered in IR muscle corresponding to intramyocellular lipid content.

A primary finding of our study was that total as well as saturated and unsaturated ceramide content was ~2-fold higher in IR skeletal muscle in human subjects independent of obesity, physical fitness and plasma free fatty acid concentration. These results extend the findings of Adams et al., who observed elevated ceramide content in skeletal muscle of obese subjects (13). Studies by Straczkowski et al., also found that ceramide accumulation in skeletal muscle was inversely related to insulin sensitivity (12, 15). They also suggested that higher ceramide content in lean offspring of patients with T2DM was due to decreased ceramide degradation, whereas IR of obesity was caused by an increase in de novo ceramide synthesis (12, 15). Not all studies, however, have observed an association between muscle ceramide content and insulin resistance (14, 19). These discrepant results could be attributed to differences in the subject population studied, e.g. age, gender, physical activity, as well as methodological differences in assessing ceramide content and insulin sensitivity (11). Thus, provides important new information about the relationship between elevated ceramide content and muscle insulin resistance This is the first ~~to~~ comprehensive profile of the content of the various molecular species of ceramide in human IR muscle using tandem mass spectrometry while controlling for obesity, physical fitness and age. This is the first study to demonstrate elevated levels of both saturated and unsaturated ceramides, as well as specific ceramides containing C14:0, C16:0, and C18:0 fatty acids in IR human muscle.

None of the prior studies examining ceramide in human muscle have accounted for

heterogeneity in muscle type that could account for differences in insulin sensitivity. Our novel finding that ceramide content was associated with a higher percentage of type II myocytes suggests that ceramide accumulation may be fiber type dependent. Further investigation is needed to confirm this. In addition, the complementary observations that both the IMTG content in type I myocytes and the lower proportion of type I myocytes are related to insulin resistance provide insight into muscle heterogeneity in lipid metabolism in insulin resistance. These data suggest a possibility that type I myofibers may have a greater capacity to buffer increases in free fatty acids by partitioning to lipid droplet IMTG, whereas type II fibers have a reduced capacity to incorporate excess fatty acids into IMTG and are more inclined to partition to lipotoxic mediators such as ceramide species. An impairment of skeletal muscle oxidative capacity has been suggested to initiate or potentiate insulin resistance (35). Our data indicate that the proportion of type I and type II myocytes is associated with higher ceramide content and insulin resistance, and that the expression levels of several genes related to mitochondrial biogenesis and fatty acid metabolism are lower in insulin resistance. Therefore, our complementary results support the concept that a low oxidative capacity of muscle may be associated with ceramide accumulation and insulin resistance. This is consistent with previous reports that the increase in whole muscle IMTG content in obesity or T2DM is primarily due to increased IMTG in type I myocytes (25), and that differences in fiber type proportion also play a role in insulin resistance of obesity and T2DM (36, 37). These observations are supported by a number of reports suggesting that type I myocytes are more insulin responsive than type II myocytes (26, 27, 38). Yet, differences in fiber type proportion between obese subjects either with or without T2DM have been reported in some (36, 37), but not all previous studies (22, 24).

Despite evidence in IR animal models supporting a role for DAG in insulin resistance (8,

9), we did not observe an increased level of DAG in insulin resistant muscle. Straczkowski et al. have also reported elevated levels of ceramide, but not DAG, in obese impaired glucose tolerant (IGT) versus obese normal glucose tolerant (NGT) subjects (12). A possible reason for lipid-induced insulin resistance by ceramide and not DAG, and *visa versa*, may be higher levels of palmitate result in greater ceramide accumulation, while unsaturated fatty acids partition towards DAG (39). Thus both ceramides and DAG can potentially induce insulin resistance depending on the degree of saturation of the lipid overload. Our finding that C16:0 ceramide was more than 3-fold higher in IR muscle is consistent with this. Thus it is plausible that basal levels of ceramide and DAG in muscle are influenced by the degree of saturation and palmitate content in the diet of obese individuals. Further studies are needed to address this attractive hypothesis.

We also examined the differential expression of several other genes relating to lipid metabolism, which could underlie accumulation of IMTG and other bioactive species, i.e., DAG and ceramide. We found that many genes involved in ceramide synthesis and degradation were not differentially expressed in IR muscle with increased ceramide content. A recent report suggests that over expression of acid ceramidase prevents the inhibitory effect of saturated fatty acids on insulin signaling (40). Also, inhibition of serine palmitoyltranseferase in zucker diabetic fatty (ZDF) rats, by treatment with the amino acid antibiotic myriocin, prevented the onset of T2DM and inhibited an increase in ceramide content in muscle, when compared to non-treated ZDF rats (41). Our results suggest that factors other than increased enzyme content, possibly increased enzyme activity or fatty acid flux, contribute to ceramide accumulation in IR muscle. In support of this, Straczkowski et al., demonstrated that the activity of neutral sphingomyelinase, the enzyme that converts sphingomyelin to ceramide, is increased in muscle of obese IGT compared to obese NGT, concomitant with higher ceramide content (12). Finally,

the observation that gene expression of TNFα tended to be elevated in IR muscle, albeit statistically non-significant, concomitant with elevated ceramide content is consistent with data demonstrating that TNFα can stimulate de novo ceramide biosynthesis (42).

To further characterize intramyocellular lipid content and metabolism in IR skeletal muscle, we determined the expression of selected genes coding for lipid droplet proteins and proteins involved in lipid droplet hydrolysis. ATGL and HSL expression/activity have been demonstrated to contribute to adipose tissue and skeletal muscle triacylglycerol lypolysis, elevated plasma fatty acid levels and accumulation of ectopic lipid deposits associated with T2DM (43). Our observation that ATGL and HSL mRNA are lower in IR muscle is in line with reports of lower expression of these lipases in obesity and/or insulin resistance (44, 45). We also found lower expression of ADRP in IR muscle. Consistent with this, Philips et al. showed that skeletal muscle protein expression of ADRP was up-regulated in response to improvements in insulin action induced by weight loss and insulin sensitizer pharmacotherapy (46). Furthermore, ATGL and ADRP have been reported to be more highly expressed in type I muscle fibers (47, 48). The fiber type-specific expression of ATGL and ADRP, and our observation of reduced type I fibers in obese IR subjects, potentially explains the lower level of ATGL and ADRP observed in whole muscle in the present study. It is tempting to speculate the same may be true for other lipases, and that fiber type composition plays a greater role in insulin resistance that previously thought. Furthermore, the trend toward higher TNFα gene expression tended to be higher in IR muscle is in line with reports that TNFα represses ATGL expression (49). We also observed for the first time in human muscle that the expression of SNAP23, a member of the SNARE family of proteins whose function is to mediate intracellular vesicle fusion events including GLUT4 transport to the plasma cell membrane and lipid droplet fusion (50), is lower in IR muscle. This

novel finding potentially linking intramyocellular lipid droplets and glucose transport is supported by others describing an association between SNAP23 and fatty acid-induced insulin resistance (50).

Future studies should be conducted to examine whether or not interventions such as exercise or weight loss affect oxidative capacity, DAG, ceramide and insulin sensitivity to better understand these associations in humans. Although we substantiated our insulin resistance phenotype with expression of key genes involved in insulin signaling and glucose transport, additional studies are needed to link intramyocellular lipids, fiber type, oxidative capacity and activation of specific insulin signaling proteins. Taken together, however, these results suggest a role for muscle fiber type proportion and/or the oxidative capacity of muscle in insulin resistance, possibly through partitioning muscle lipids into either triglycerides or ceramides.

In summary, ceramide but not diacylglycerol is elevated in IR human skeletal muscle independent of obesity, physical activity or aerobic fitness. Moreover, ~~this study also indicates that~~ muscle fiber type as well as fiber type-specific lipid content is associated with insulin resistance in muscle. Importantly, key genes associated with fatty acid metabolism and lipid droplet regulation are decreased in IR muscle. Together, these data suggest that mechanisms underlying the associations between intramyocellular lipids and insulin resistance likely involve ceramide, which in turn may be dependent on the muscle fiber type or its oxidative capacity. Future mechanistic studies are needed to further elucidate these important findings.

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TABLE 1. Group characteristics

	Insulin Sensitive	**Insulin Resistant**
n	10	12
Age, yr	64.5 ± 1.4	66.7 ± 0.82
Body mass, kg	81.1 ± 3.0	81.7 ± 2.5
BMI, kg/m^2	30.3 ± 1.1	31.3 ± 0.9
VO_2peak, ml/kgBW/min	15.8 ± 1.1	15.5 ± 1.0
Fasting plasma insulin, mU/ml	3.7 ± 0.5	6.5 ± 1.3
Fasting plasma glucose, mg/dl	94.1 ± 5.1	96.7 ± 5.0
Fasting plasma FFA, μM	502.4 ± 41.2	543.3 ± 41.7
OGTT Glucose @ 120min, mg/dl	122 ± 10	164 ± 12*
R_d, ml/kgFFM/min	10.2 ± 0.3	7.1 ± 0.4*

Data are means ± SEM. BW, body weight. FFM, fat free mass. FFA, free fatty acid. * = Significantly different from IS group, $P < 0.05$

TABLE 2. Muscle fiber type distribution and surface area in vastus lateralis muscle

	Insulin Sensitive Group	**Insulin Resistant Group**
Type I myocyte content, %	59±3.9	41±2.5*
Type II myocyte total content, %	40.5±3.9	58±2.9*
**Type IIa myocyte content, %	24±2.7	34±1.9*
**Type IIx myocyte content, %	14±2.5	25±3.4*
Type I myocyte average area, μm^2	5732±317	5104±417
Type II myocyte average area, μm^2	4217±348	4027±375

Data are means ± SEM. * = Significantly different from IS group, $P < 0.05$.

** = Data generated from analysis of subgroup, (IR, n=10; IS, n=5)

TABLE 3. Correlation of Rate of Glucose Disposal (ml/kgFFM/min) with other study variables when data from both IR and IS groups were combined.

	Pearson Correlation Coefficient
Type I Myocyte content, %	** 0.608
Type II Myocyte total content, %	**-0.724
Total Ceramide, pMol/mg protein	**-0.563
Total Saturated Ceramide, pMol/mg protein	**-0.567
Total Unsaturated Ceramide, pMol/mg protein	*-0.438
Total Diacyglyceride, pMol/mg protein	0.006
ADRP gene expression, $2^{-\Delta Ct}$	** 0.810

* = Correlation is significant at the level $P < 0.05$

** = Correlation is significant at the level $P < 0.01$

FIGURE 1: Sphingolipid (Panel A) and diacylglycerol (Panel B) content in vastus lateralis of IR and IS subjects. IR data is expressed relative to IS group, which was set to a value of 1. * = Significantly different from IS group, $P < 0.05$.

FIGURE 2: Myocyte-specific IMTG content in vastus lateralis of IR and IS groups. Panel A; representative image of Oil red O stain (20x objective). Panel B; representative image of immunostain for fiber type (20x objective). Panel C; Pearson correlation of R_d with type I myocyte-specific IMTG content. White data points = IS subjects, Black data points = IR subjects. Panel D; Pearson correlation of R_d with type II myocyte-specific IMTG content. White data points = insulin sensitive, Black data points = insulin resistant. Panel E; IMTG content in type I myocytes, type II myocytes, and total IMTG in vastus lateralis from IR and IS groups. * = Significantly different from IS, $P < 0.05$.

FIGURE 3: Gene expression in vastus lateralis of IR and IS sub-groups. IR gene expression is expressed relative to IS group, which was set to a value of 1. Black bar = IS group. White bar = IR group. TNF, Tumor necrosis factor. PGC, Peroxisome proliferator-activated receptor gamma coactivator. PPAR, Peroxisome proliferator-activated receptor. NRF, Nuclear respiratory factor. ACAC, Acetyl-Coenzyme A carboxylase. MLYCD, Malonyl-CoA decarboxylase. CPT, Carnitine palmitoyltransferase. SNAP, Synaptosomal-associated protein. ATGL, Adipose triglyceride lipase. ADRP, Adipose differentiation-related protein. HSL, Hormone sensitive lipase. S3-12, Plasma membrane associated protein KIAA1881. CERK, Ceramide kinase. ASAH, N-acylsphingosine amidohydrolase. SPTLC, Serine palmitoyltransferase, long chain

base. ABHD5, Abhydrolase domain containing 5. GLUT4, Facilitated glucose transporter member 4. IRS, insulin receptor substrate. * = Significantly different from IS group, $P < 0.05$.

FIGURE 1.

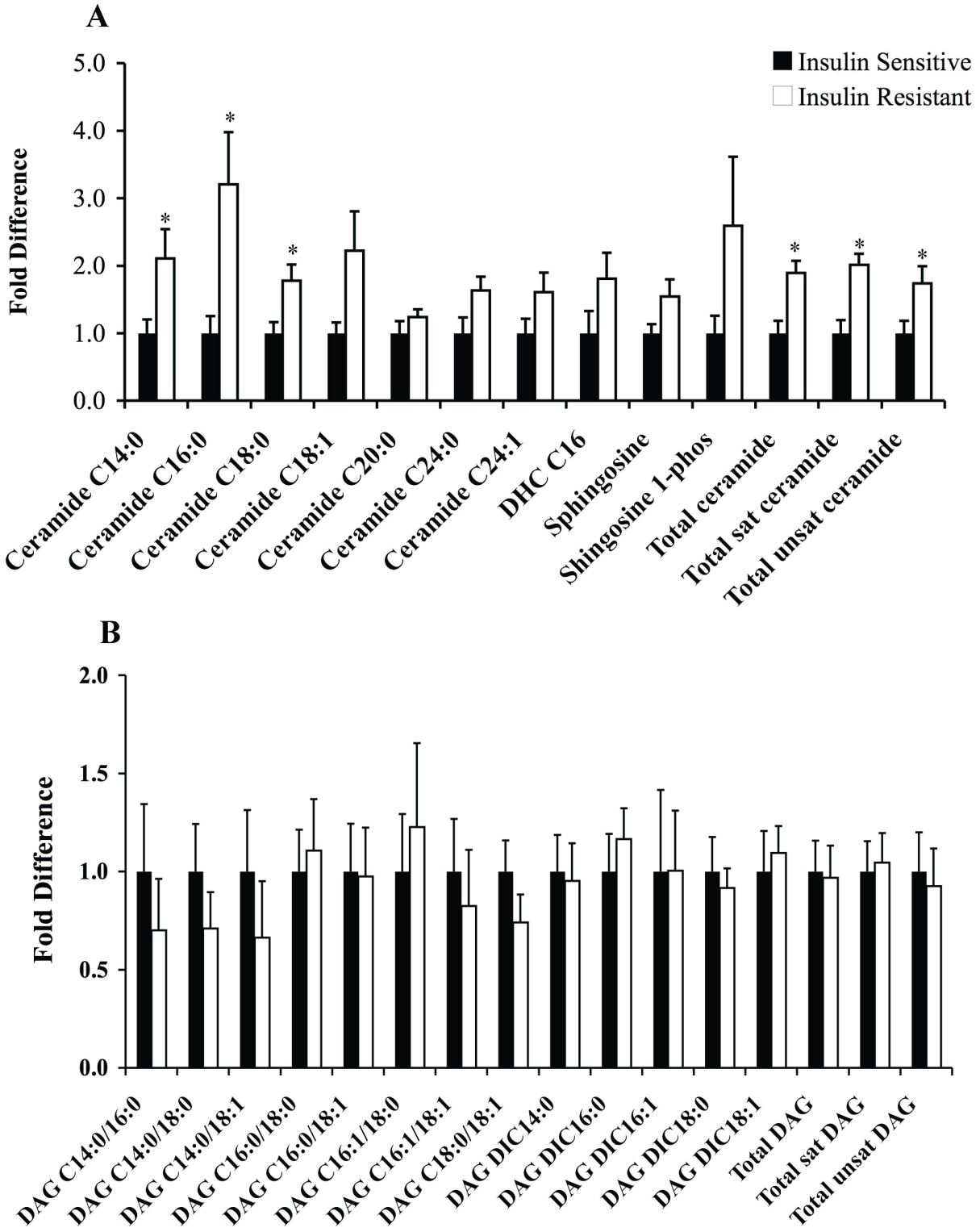

A
Insulin Sensitive
Insulin Resistant
Fold Difference
5.0
4.0
3.0
2.0
1.0
0.0
*
Ceramide C14:0
Ceramide C16:0
Ceramide C18:0
Ceramide C18:1
Ceramide C20:0
Ceramide C24:0
Ceramide C24:1
DHC C16
Sphingosine
Shingosine 1-phos
Total ceramide
Total sat ceramide
Total unsat ceramide
B
Fold Difference
2.0
1.5
1.0
0.5
0.0
DAG C14:0/16:0
DAG C14:0/18:0
DAG C14:0/18:1
DAG C16:0/18:0
DAG C16:0/18:1
DAG C16:1/18:0
DAG C16:1/18:1
DAG C18:0/18:1
DAG DIC14:0
DAG DIC16:0
DAG DIC16:1
DAG DIC18:0
DAG DIC18:1
Total DAG
Total sat DAG
Total unsat DAG

FIGURE 2.

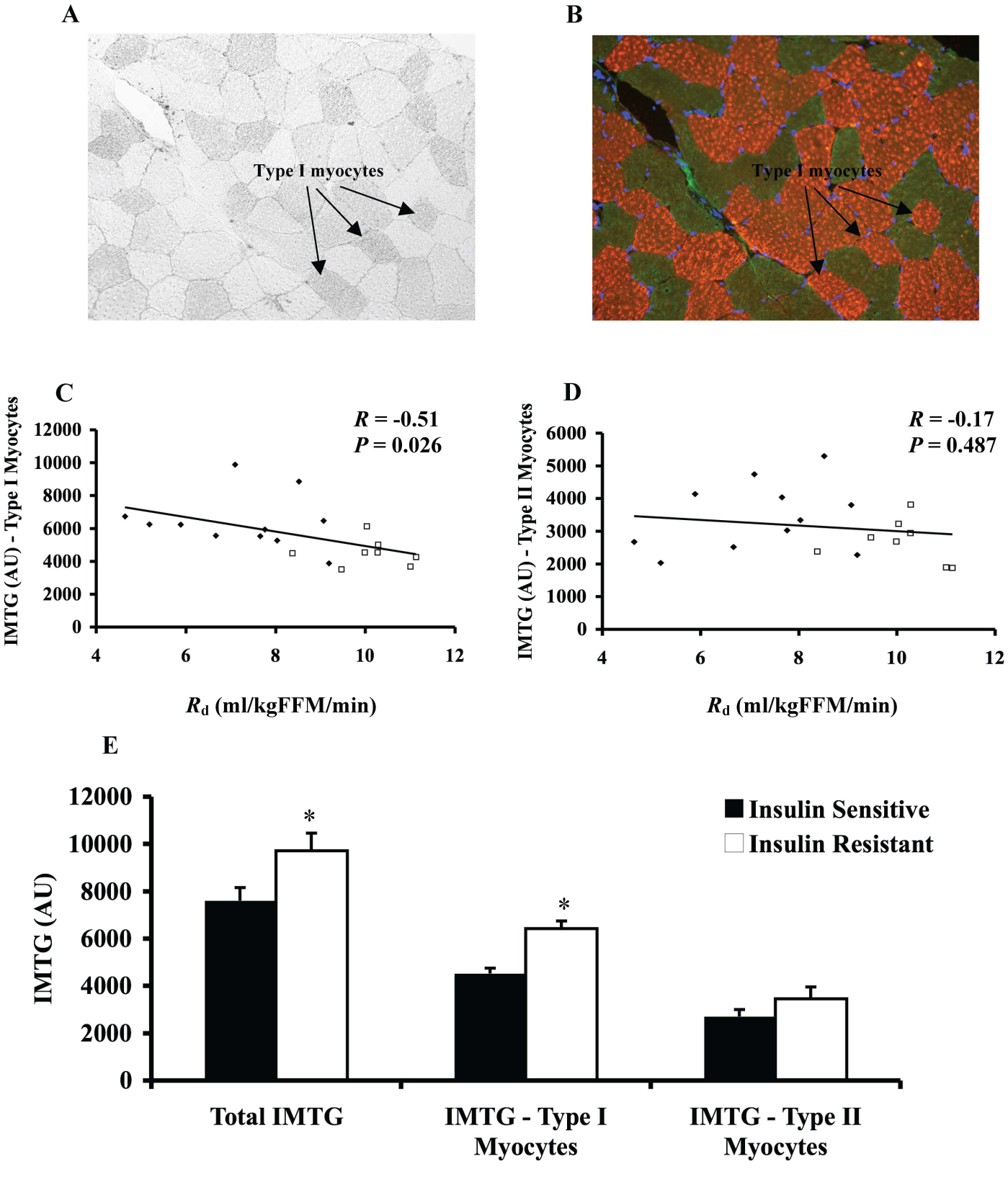
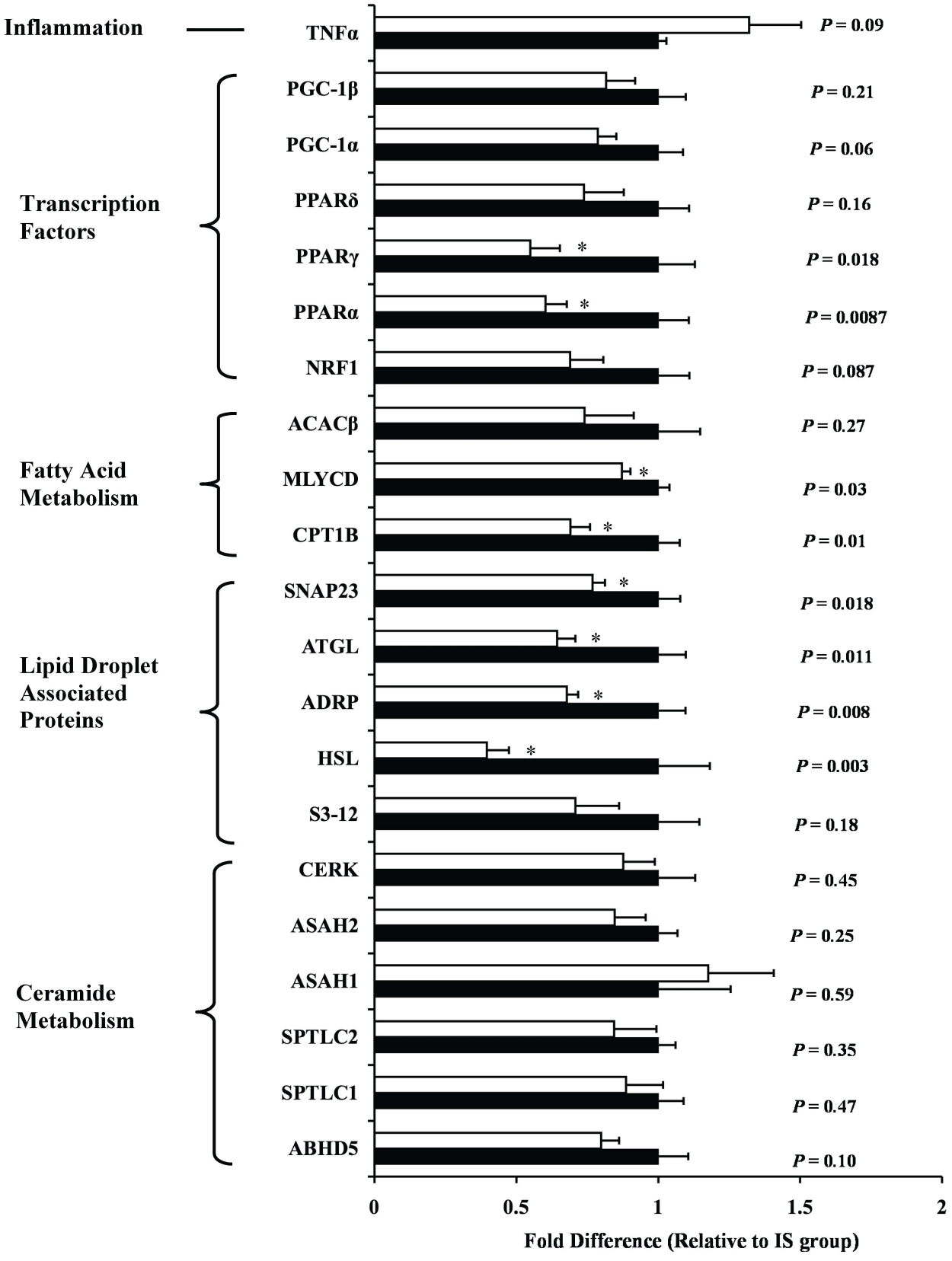

A
Type I myocytes
B
Type I myocytes
C
R = -0.51
P = 0.026
IMTG (AU) - Type I Myocytes
12000
10000
8000
6000
4000
2000
0
4
6
8
10
12
Rd (ml/kgFFM/min)
D
R = -0.17
P = 0.487
IMTG (AU) - Type II Myocytes
6000
5000
4000
3000
2000
1000
0
4
6
8
10
12
Rd (ml/kgFFM/min)
E
IMTG (AU)
12000
10000
8000
6000
4000
2000
0
*
*
Insulin Sensitive
Insulin Resistant
Total IMTG
IMTG - Type I Myocytes
IMTG - Type II Myocytes

FIGURE 3.


Inflammation
TNFα
P = 0.09
Transcription Factors
PGC-1β
P = 0.21
PGC-1α
P = 0.06
PPARδ
P = 0.16
PPARγ
*
P = 0.018
PPARα
*
P = 0.0087
NRF1
P = 0.087
Fatty Acid Metabolism
ACACβ
P = 0.27
MLYCD
*
P = 0.03
CPT1B
*
P = 0.01
Lipid Droplet Associated Proteins
SNAP23
*
P = 0.018
ATGL
*
P = 0.011
ADRP
*
P = 0.008
HSL
*
P = 0.003
S3-12
P = 0.18
Ceramide Metabolism
CERK
P = 0.45
ASAH2
P = 0.25
ASAH1
P = 0.59
SPTLC2
P = 0.35
SPTLC1
P = 0.47
ABHD5
P = 0.10
0
0.5
1
1.5
2
Fold Difference (Relative to IS group)